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Laboratory Protocol

Protocol number: 4
Protocol description: Construction of partial genomic library enriched for microsatellite repeats
Original references: Kandpal et al (1994); Mundy & Woodruff (1996), Lunt et al. 1999, Iyengar et al 2000
Original entry: A. Iyengar, December 2001
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We use a biotin-capture based enrichment procedure, which is outlined in Figure 1

Required materials:
starred (*) reagents are described in the 'standard laboratory solutions' document)

1. MboI restriction enzyme (Promega or other)
2. Agarose (any Molecular Biology Grade)
3. 10X TBE buffer, pH 8.3 (108g Tris, 55g Boric acid, 9.3g EDTA in 1 litre water)
4. 100 bp ladder
5. Qiaquick PCR and gel purification columns (Qiagen)
6. MboI adapter oligos (PAGE purified)
    1. 5'pGATCGCAGAATTCGCACGAGTACTAC
    2. CGTCTTAAGCGTGCTCATGATGC
7. TE buffer (10mM Tris, 1mM EDTA, pH 8.0)
8. T4 DNA ligase (Promega or other)
9. LB-agar
10. LB medium
11. Ampicillin
12. Grids made out of transparent film cut to 90mm petridish size
13. 90mm petridishes
14. Toothpicks and plastic/glass spreaders
15. 50mg/ml X-gal and 0.2M IPTG for blue/white screening
16. 96-well plates for colony preservation and PCRs
17. Primers for PIMA
    1. M13FOR - GTTTTCCCAGTCACGAC
    2. M13REV – CAGGAAACAGCTATGAC
    3. CA RPT PRIMER - TGTGGCGGCCGCTGTGTGTGTGTGTGTGV respectively
18. Qiaprep columns for plasmid purification (Qiagen)
19. ABgene Thermoprime plus DNA polymerase (or other ordinary Taq polymerase). Use of ABgene 10X Reddy Mix buffer allows direct loading of an aliquot of the PCR reaction onto a gel which is particularly useful when screening colonies
20. Biotinylated oligo. e.g., for CA enrichment:
    5'biotin-ATAGAATATCACACACACACACACACACACACACACACACACACACACACA

Required materials (continued):

21. 1.0M Sodium phosphate buffer, pH 7.4 (mix 3.096mls 2.5MNa_2HPO_4, 0.904ml 2.5M NaH_2PO_4, 6mls sterile distilled water)
22. 10% Sodium dodecyl sulphate (SDS)
23. Streptavidin coated beads (Promega)
24. 1x Tris buffer (100mM Tris pH 7.5, 150mM NaCl)
25. 0.1x Tris buffer
26. Sterile distilled water
27. pGEM-T Easy vector + JM109 competent cells (Promega)
28. dNTPs, dATP

Required equipment:

1. Spectrophotometer/fluorometer
2. Agarose electrophoresis equipment
3. Scalpel blades
4. Heat block (or use PCR machine)
5. PCR machine
6. Magnetic particle concentrator (Promega)
7. Bench centrifuge
8. Basic pipettes and multi-channel pipettes

Protocol:
1. Extract high quality genomic DNA using the tissue available from the organism of interest, using any standard protocol.

2. Quantify the DNA using a spectrophotometer/fluorometer – you will need 10-20µg per enrichment.

3. Restrict the DNA with MboI overnight at 37ºC using ~30 units enzyme in a total volume of about 50µl depending on the concentration of the DNA. Run a small aliquot to confirm restriction and then electrophorese entire restricted sample on a 1% agarose gel along with a marker such as 100bp ladder.

4. Cut out 300-1000bp sized DNA smear from the gel using a scalpel blade. Purify DNA from gel using Qiaquick columns (Qiagen) and quantify recovered DNA once again.

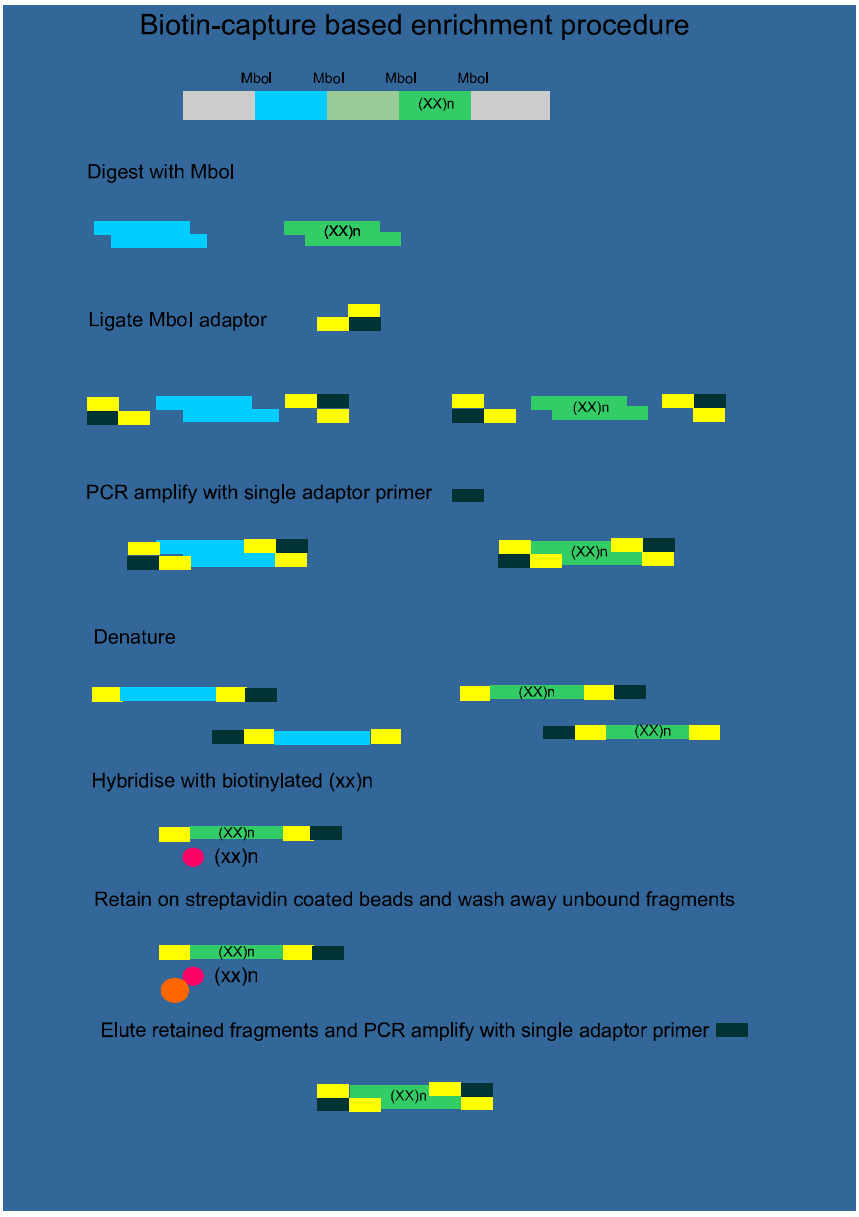

Figure adapted from Kandpal et al. (1994).

5. Formation of a MboI adapter oligo:

Dissolve the two adapter oligos at a concentration of 250µM in TE buffer. Pre-anneal them by taking equimolar amounts of oligos supplemented with 100mM NaCl in a tube and placing the tube in a heat

cool slowly to room temperature over 30-40 minutes. The adapter oligo can then be used for the ligation step or stored at -20ºC until use.

6. Ligation of MboI adapter molecule to MboI digested genomic DNA:

Use 2.5-5µg of genomic DNA and 7.5-15µg adapter oligo and ligate using ~6 units of T4 DNA ligase overnight at 16ºC.

Purify ligated products through Qiaquick columns (Qiagen) into a volume of 50µl. Use 1µl of the recovered DNA in a 50µl PCR reaction with either of the two adapter oligos as a primer using the following conditions: 1x 94°C, 4 min; 35x 94°C, 30 sec, 55°C, 30 sec, 72°C, 120 sec; 1x 72°C, 10 min.

Check an aliquot of the PCR reaction by 1% agarose gel electrophoresis to confirm the appearance of a smear between 300-1000bp. Purify products once again through Qiaquick columns and quantify recovered DNA. You will need a minimum of 2µg for the hybridisation step.

7. Hybridisation to biotinylated oligos:

Resuspend biotinylated tandem repeat oligo at 2µg /µl. Use 2-4µg PCR product and 1-2µg biotinylated oligo for hybridisation. Denature PCR product first by placing at 94ºC for 10 minutes and then placing on ice. Then add sodium phosphate buffer (pH7.4) to a final concentration of 0.5M, the biotinylated oligo and SDS to a final concentration of 0.5%. Hybridisation is carried out overnight at 50ºC in a volume of 50-100µl.

8. Enrichment:

Take 1 tube of streptavidin coated beads (0.6ml) and wash 3 times with 1X Tris buffer. Each time, the magnetic particle concentrator is used to remove solution by pipetting, while the beads adhere to the side of the tube which is in contact with the magnetic concentrator. Then add the entire hybridisation mixture and incubate at room temperature for 30minutes. For the enrichment, a series of washes are carried out at increasing stringency, thus washing away a proportion of un-hybridised DNA at every step. The magnetic particle concentrator is used at every step to remove solutions, leaving behind beads with bound DNA.

The washes for $(CA)_{20}$ enrichment are listed below. Each wash solution is incubated with the beads for a period of 15 minutes.

1) 1X Tris buffer – room temperature
2) 1X Tris buffer - 50ºC
3) 0.1X Tris buffer - 50ºC
4) 0.1X Tris buffer - 65ºC
5) Sterile distilled water - 65ºC

DNA from all the washes (or from only the last two washes – see below*) is purified using Qiaquick columns and amplified in PCR reactions as described above (step#6).

Note: A Southern blot of the gel followed by hybridisation to a CA repeat probe can be carried out at this stage to check for enrichment and confirm that the last wash is in fact the mostly highly enriched for CA repeats. We have found that in some cases, wash 4 can be more highly enriched than the final wash no. 5.

*The alternative is to clone DNA obtained from both wash 4 and wash 5 separately and screen for repeat containing clones using the PCR based technique PIMA (see below) in each case.

Quantify all purified PCR products and set up ligations for cloning. Cloning can be done into any commercially available vector such as pBluescript after restriction of the genomic DNA and vector with MboI followed by alkaline phosphatase treatment of the vector. An alternative that we follow is to

clone the DNA into pGEM-T Easy vector (Promega) after PCR and an extended A-tailing step as follows:

After completion of PCR, add an additional 0.2mM dATP and 0.2U Taq polymerase and incubate at 72ºC for 15 minutes. Then purify DNA through Qiaquick columns and quantify for ligation to vector.

9. Cloning:

Clone genomic DNA into pGEM-T Easy following manufacturer's protocols. We use 100ng vector with a 3:1 insert: vector ratio i.e. in the case of a range of insert fragments of 300-1000bp, you will need 65ng of insert DNA for 100ng of vector (3kb).

Plate out 100µl of transformed culture onto 2 plates containing LB-agar medium + ampicillin (100µg/µl final concentration), having spread 50µl of 200mM IPTG and 20µl of 50mg/ml X-gal on the surface of the plates and allowed to dry. Allow colonies to grow overnight at 37ºC. White colonies containing inserts are then picked for screening. We use a PCR based procedure PIMA (Lunt et. al, 1999) for screening for repeat-containing clones as follows:

Pick individual white colonies using a toothpick, spot onto a gridded plate (to enable subsequent identification) and swirl in 50µl sterile distilled water within the corresponding well of a 96-well plate, again allowing subsequent identification. We pick 188 colonies from each agar plate (2 x 96 well plates + 2 negative controls with water alone on each plate) i.e. a total of 376 colonies from each enrichment. Each 96-well plate is placed at 94ºC for 15 minutes and stored at -20ºC until use. The gridded plate is placed at 37ºC overnight for the colonies to grow and then placed at 4ºC (tightly wrapped with parafilm to reduce desiccation) until the selected repeat-containing cultures can be grown.

PIMA-PCRs are then performed within 96-well plates using 1µl each from the colony containing 96-well plate as follows: a 20µl reaction is set up using three primers (M13forward, M13reverse, and CA-repeat primer). The PCR conditions are as follows:
X1 - 94ºC, 4mins; X30 - 94ºC, 30 secs, 55ºC, 60 secs, 72ºC, 60 secs; X1 - 72ºC, 10mins.

The PCR products are then run on a 1% agarose gel – the presence of a smaller band in addition to the band produced by amplification across the insert using M13for and rev primers is an indication of the presence of a CA repeat within the insert. We have found that the presence of a smeary/stuttery band in addition to the insert band is a better indication of a CA repeat than the presence of an extra band itself as described in Lunt et al. (1999). We find frequent appearance of an extra band as a result of non-specific hybridisation of the repeat primer.

NOTE: The use of multi-channel pipettes and a multi-channel-pipette-friendly gel system greatly speeds up this process of screening. We have also recently started using the ABgene Thermoprime Plus DNA polymerase with 10X Reddy Mix PCR buffer which allows direct loading of an aliquot of the PCR reaction onto a gel.

Grow the clones that are identified as positive from PIMA by picking the colony off the gridded plate and inoculating it into 3mls of LB+ampicillin and growing at 37ºC overnight in a shaker. A glycerol stock of the culture can be made for future use by mixing 0.85ml of culture with 0.15ml of sterile glycerol in a tube, vortexing the tube, and freezing down the tube at -80ºC. The plasmid DNA is then purified using Qiaprep columns (Qiagen) and sequenced using M13for or M13rev as a primer.

The sequences are then checked for the presence of repeats and primers designed. Sequencing of the opposite strand can be done as required, in cases where there are long inserts there is need for confirmation of sequence.

Note: Remember to delete adaptor oligos sequences prior to primer design if you use the PCR product for cloning into pGEM-T without restriction with MboI!

Although various levels of enrichment can be achieved with this technique and the results also depend on the species being used, we routinely find that a minimum of 10-15% of screened clones contain CA repeats. A second enrichment step of the genomic DNA after the first enrichment step may help in increasing these numbers.

We are currently trying to optimise the entire procedure for a number of tetranucleotide and trinucleotide repeats.

References:

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